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Award Number: W81XWH-13-1-0065

TITLE: A Biochemical Approach to Understanding the Fanconi Anemia Pathway-Regulated Nucleases in Genome Maintenance for Preventing Bone Marrow Failure and Cancer

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REPORT DATE: April 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
May 2014	Annual	1 May 2013 – 30 April 2014

4. TITLE AND SUBTITLE
A Biochemical Approach to Understanding the Fanconi Anemia Pathway-Regulated Nucleases in Genome Maintenance for Preventing Bone Marrow Failure and Cancer

5a. CONTRACT NUMBER
W81XWH-13-1-0065

5b. GRANT NUMBER
W81XWH-13-1-0065

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Rockefeller University
1230 York Ave
New York,
NY, 10065

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Fanconi anemia is the most prevalent inherited BMF syndromes, caused by mutations in at least 16 genes. A hallmark of FA is cellular hypersensitivity to agents that form interstrand cross-links (ICLs). The FA pathway maintains genome stability by co-ordinating the necessary repair response required for the full removal of ICLs. However, the specific function of FA proteins and associated factor remain a very important puzzle to solve. Failed or inappropriate attempts to repair ICL lesions will result in genomic instability that has been postulated to be the genetic causes of both BMF and subsequent cancer development in FA patients. The objective of the proposed project is to develop biochemical systems to characterize the molecular details of ICL repair involved in genome maintenance. Comprehension in such molecular mechanisms will contribute to elucidating both the cause for initiation and step-wise transformation of BMF syndromes to cancer.

15. SUBJECT TERMS
Fanconi anemia, nucleases, bone marrow failure, interstrand cross-link repair

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Fanconi anemia (FA) is one of the most prevalent inherited bone marrow failure (BMF) syndromes, caused by mutations in at least 16 different genes that are implicated in a common pathway important for genome maintenance (1,2). A hallmark of FA is cellular hypersensitivity to chemotherapeutic agents that form interstrand cross-links (ICLs). Endogenous ICLs can also arise as a result of cellular metabolism. It is thought that the FA pathway maintains genome stability by co-ordinating the necessary repair response required for the full removal of ICL lesions. Dual incision around the ICL represents a pivotal step in initiation of the repair process. A number of nucleases have been suggested to perform this critical incision event, including XPF-ERCC1 (3-6), MUS81-EME1 (7), SLX1-SLX4 (8-10), and the Fanconi anemia Associated Nuclease 1 (FAN1) (11-14). How these nucleases are co-ordinated by the FA pathway is a very important puzzle to solve, since failed or inappropriate attempts to repair ICL lesions will result in genomic instability with increased radial formations and wide range of chromosomal abnormalities as observed in cells derived from FA patients (15). The purpose of the proposed project is to develop biochemical systems to characterize the molecular details of ICL repair involved in genome maintenance that prevents bone marrow failure and malignant transformation.

2. KEYWORDS

Fanconi anemia, nucleases, interstrand cross-link repair

3. OVERALL PROJECT SUMMARY

Aim 1: To explore the relationship between the nuclease proteins implicated in interstrand cross-link repair by using in vitro systems.

Task 1: Further purification of proteins used in this study.

Both wildtype (WT) and nuclease-dead mutant (MUT) of his-tagged FAN1 were expressed in sf9 cells and purified by Ni-NTA purification (Figure 1a). Recombinant FAN1 obtained from this purification appears to be active, showing strong endonucleolytic activity. However, even in the mutant mutant FAN1 prep there is strong exonucleolytic activity (Figure 1b), indicating the presence of a contaminating exonuclease in our protein prep.

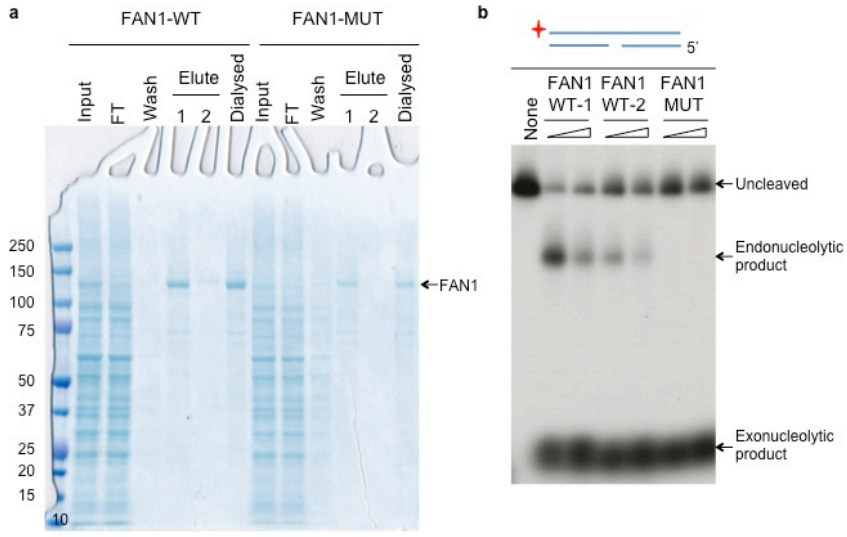

Figure 1. Purification and incision assay of FAN1. (a) Coomasie-stained gel showing Ni-NTA purification steps of his-tagged FAN1 expressed in sf9 cells. Both wildtype (WT) and nuclease-dead mutant (MUT) FAN1 were purified in parallel. (b) Incision assay was performed by incubating recombinant human FAN1 with nicked linear DNA duplex radioactively 5' ^{32}P labeled on the strand indicated with a red asterisk. Reaction products after 30 minute incubation were subjected to denaturing PAGE.

Ion exchange chromatography was employed to purify the contaminant away from recombinant FAN1. The protein preps were further purified by using open columns with Q sepharose or SP sepharose beads, and nucleolytic activitiy of each fraction was examined (Figure 2). SP sepharose beads allowed some

degree of separation of FAN1 from the contaminant (Figure 2e and 2f). However, there is partial overlap between FAN1 endonucleolytic activity and exonuleolytic activity from contaminant. This indicates that a purification system that allows greater peak resolution is required to completely purify contaminating exonuclease away.

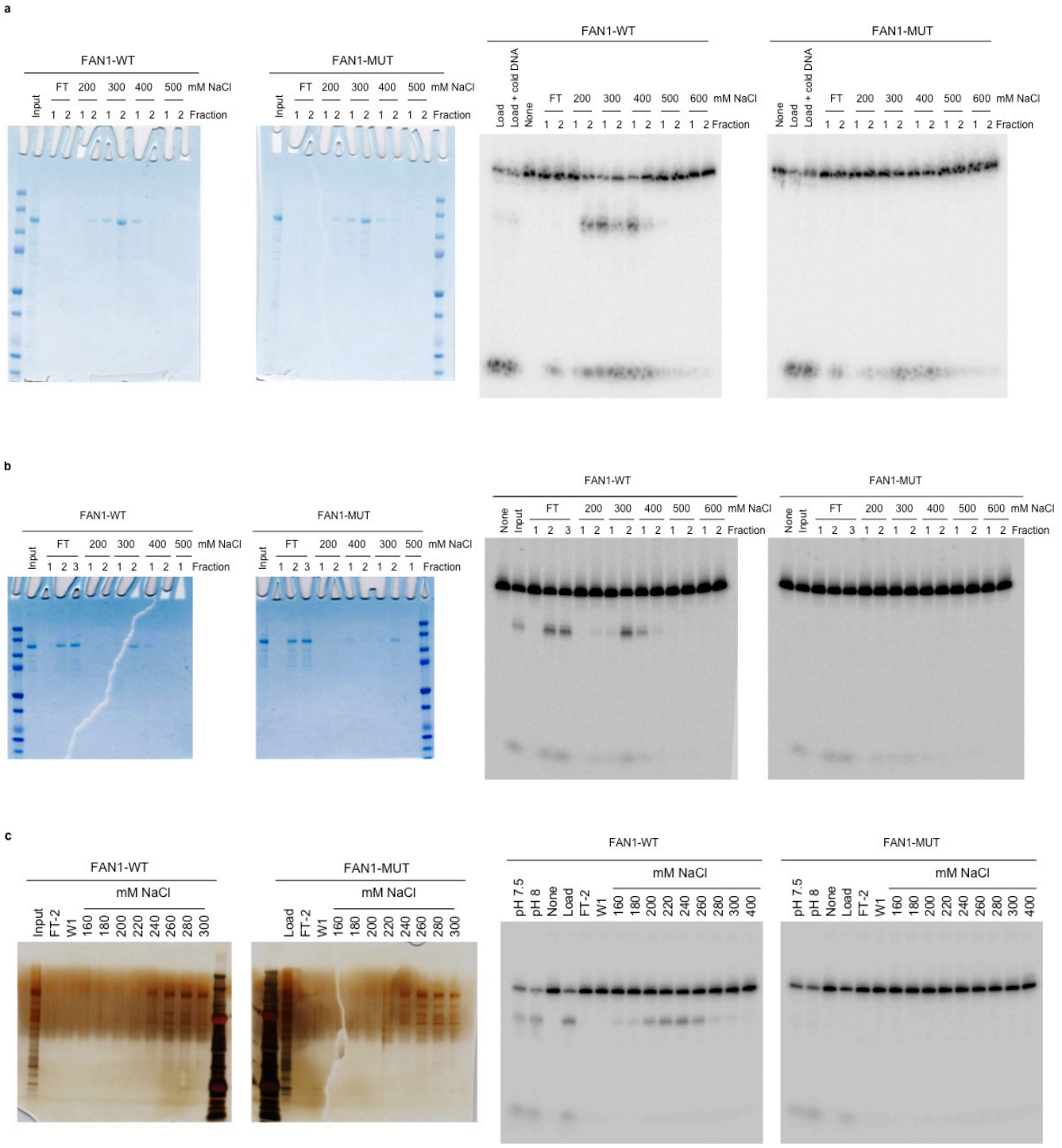

Figure 2. Purification of FAN1 by ion exchange chromatography. Recombinant FAN1 obtained from Ni-NTA shown in Figure 1 was further purified through Q-sepharose (a), SP-sepharose (b) then Q-sepharose (c) columns, and the input, flow through (FT) and each of the eluted fractions by increasing salt concentration were subjected to incision assays as described in Figure 2b. The left-hand panels show coomassie or silver-stained protein gels, whereas the right-hand panels show corresponding results from the incision assays.

FLAG-tagged SLX4 was co-expressed with each of the three associating nucleases, SLX1, XPF-ERCC1 or MUS81-EME1 in sf9 cells and purified by anti-FLAG purification (Figure 3). Both WT and MUT nucleases (Figure 3a and 3b respectively) in complex with SLX4 were purified. A significant amount of SLX4-complexes overexpressed in sf9 cells is found in the insoluble fraction, reducing the yield of soluble proteins. However, the purified protein complexes showed strong activity in nanogram scale (Figure 3c). Similar to recombinant FAN1 purification, proteins obtained from this one-step anti-FLAG purification appear to be contaminated by exonucleases as the mutant preps also show strong exonucleolytic activity albeit being endonuclease-dead (Figure 3c). We also employed ion exchange chromatography to test for conditions to further purify SLX4-nuclease complexes. FLAG-SLX4 alone bound strongly to Q-sepharose beads and eluted at high salt concentration (Figure 4), suggesting that this anion-exchange matrix is an appropriate downstream step for further purification. Indeed, when we applied FALG-SLX4-HALO-tagged SLX1 through Q sepharose column, much of the exonucleolytic activity was purified away from the main fractions that contain endonycleolytic activity (Figure 4C and 4D).

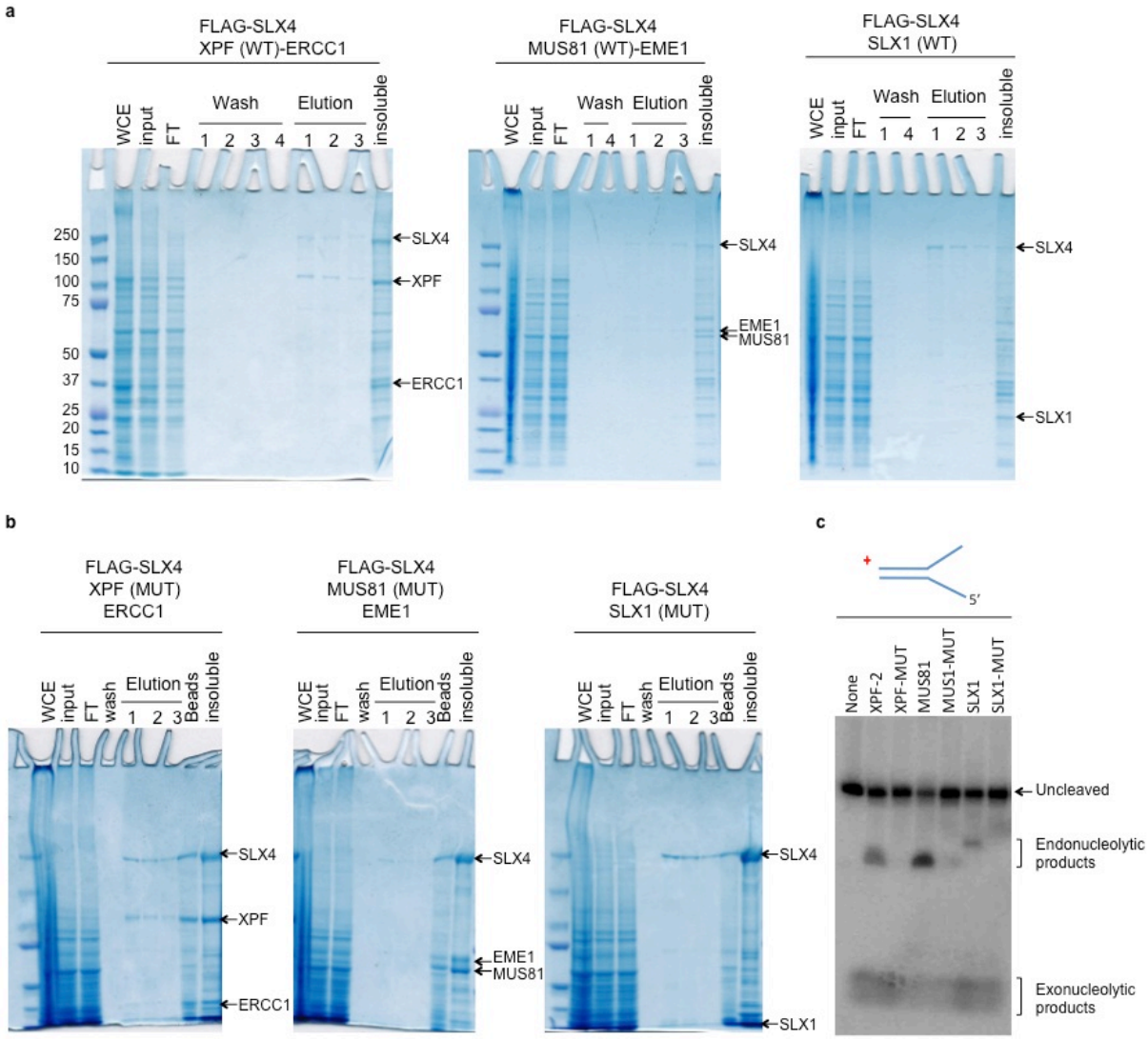

Figure 3. Purification and incision assay of SLX4-associated nucleases. SLX4-associated with WT (a) and MUT (b) nucleases were purified by using anti-FLAG M2 resins. (c) Results from incision assay using recombinant SLX4 nuclease complexes.

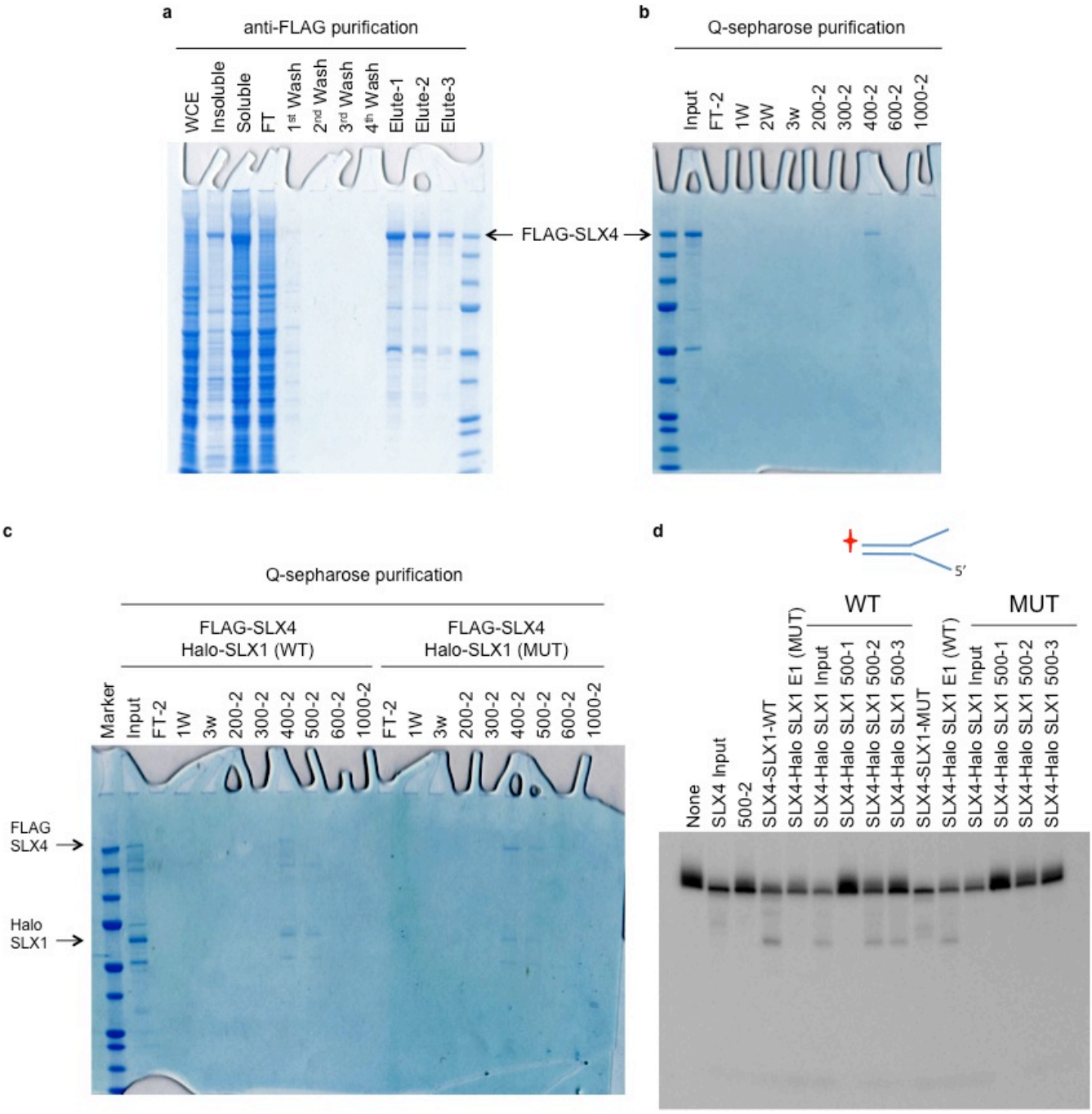

Figure 4. Purification of SLX4 by using Q-sepharose column. FLAG-tagged SLX4 alone was first affinity-purified (a), then subjected to Q-sepharose column (b). and MUT (b) nucleases were purified by using anti-FLAG M2 resins. FLAG-SLX4-HALO-SLX1 was further purified through Q-sepharose column (c), and incision assay was performed on the eluted fractions (d).

We have recently acquired an FPLC machine through the NIH R01 grant. The FPLC machine is currently being utilized to achieve better separation of proteins in each ion exchange chromatography step. Indeed, the first FAN1 prep using the FPLC machine appears to be free of the contaminating exonuclease activity. The FPLC machine will also enable us to perform gel filtration chromatography as an additional strategy for purification, which will be particularly useful for the purification of SLX4-nuclease complexes.

Task 2: The generation of DNA interstrand cross-link substrates.

DNA oligos containing modified bases for generating site-specific ICL were synthesized by Dr Ouathek Ouerfelli at the Memorial Sloan Kettering Cancer Center. The DNA oligos were designed to have different modifications at the 5' or 3' ends to allow radioactive labeling of the substrates once they are cross-linked. The oligo synthesis was initially delayed for several months due to the interference of modified bases with oligo extension and end modification. After extensive optimization, we have determined the optimal click chemistry conditions required for forming cross-links between the two oligos. By using these conditions, we are able to produce DNA ICL substrates at high efficiency that are refractory to heat denaturation (lanes 17, 19-23, 26 and 28), whereas non-crosslinked substrates that were annealed were denatured (lanes 16, 25 and 27) (Figure 5). We have successfully made linear DNA duplexes (lane 2) and splayed arm (lane 4) substrates containing a single ICL. Complementary oligonucleotides are annealed to the splayed arm ICL substrates to generate 5' flap, 3' flap and replication fork substrates. We also generated splayed arm ICL substrates with ICL being placed several nucleotides away from the junction (lanes 4-8).

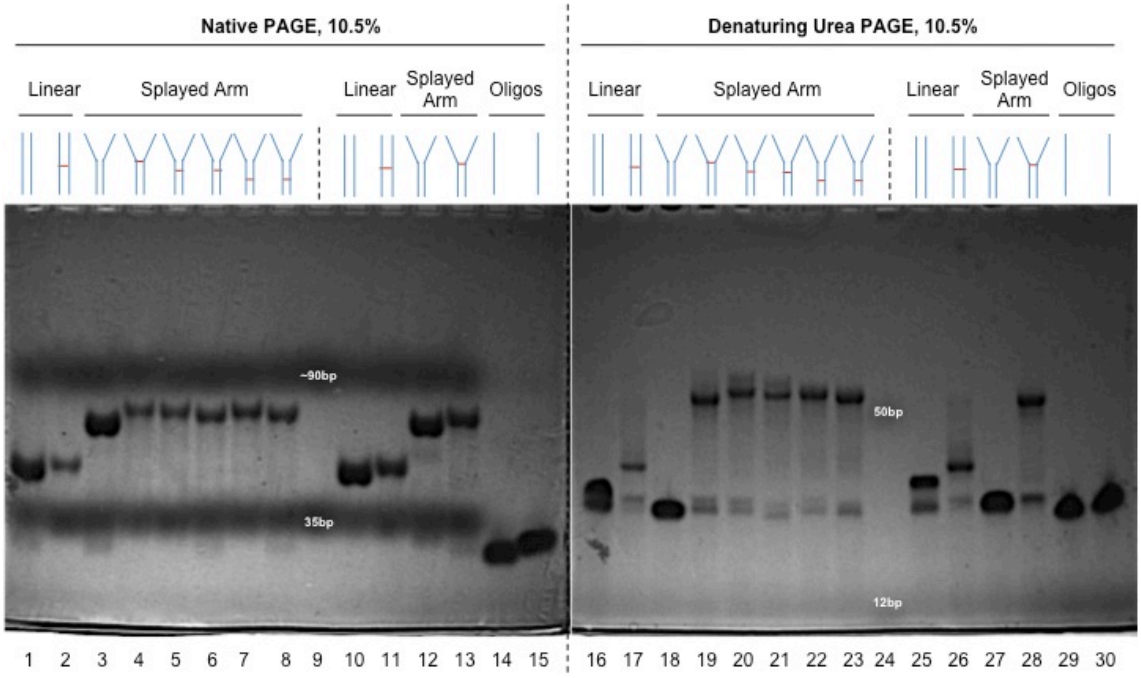

Figure 5. DNA substrates containing a site-specific interstrand cross-link. DNA oligos with modified bases were annealed and cross-linked by click chemistry. The cross-linked substrates were indicated in the schematics with a red line. Non-crosslinked oligos that were annealed were included as a control for denaturation. All substrates made were analysed by native and denaturing PAGE.

Task 3: Analysis of incision activity of nucleases.

By using nucleases purified from one step affinity purification as described in task 1, *in vitro* cleavage assays were performed on DNA substrates with different fork configurations to profile their substrate preferences. Recombinant FAN1 displayed strong activity on a wide range of substrates including nicked linear DNA substrates, replication fork and 5' flap substrates (Figure 6). The intrinsic exonucleolytic activity of FAN1 is stronger toward single stranded DNA than double stranded DNA as more of the ssDNA products generated from endonucleolytic digestion of 5' flap substrates were degraded than the dsDNA products from replication fork substrates.

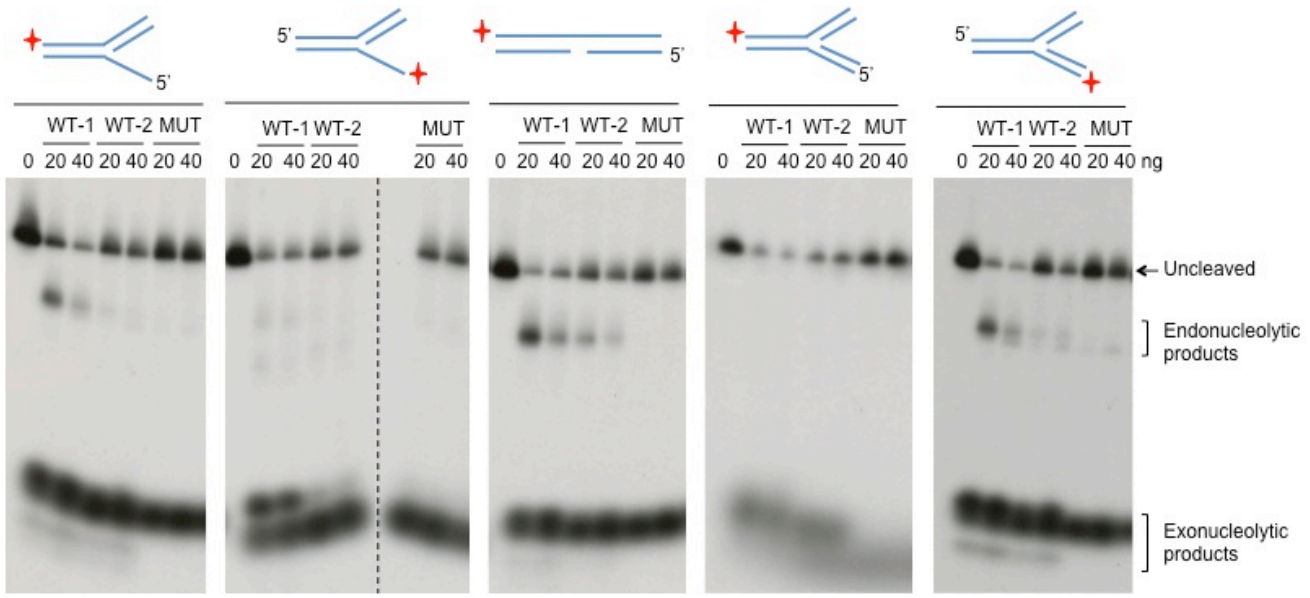

Figure 6. FAN1 showing structure-specific incision activity. Recombinant FAN1 from affinity purification was incubated with indicated substrates drawn.

The activities of purified SLX4-associated nucleases on different DNA substrates were also profiled (Figure 7). All SLX4-associated nucleases displayed activity toward the top strand of splayed arm substrates with SLX4-MUS81-EME being most active. Whereas SLX4-XPF-ERCC1 only displayed activity toward splayed arm substrate, SLX4-MUS81-EME1 showed strong activity toward the top strands of 3' flap, replication fork and 5' flap. SLX4-SLX1 is active on the bottom strands of 5' flap and splayed arm substrates and the top strands of 5' flap and splayed arm substrates. None of the SLX4-associated nucleases showed activity toward the bottom strands of 3' flap and replication fork substrates. Figure 8 provides a schematic summary of the substrate specificities of the different nucleases.

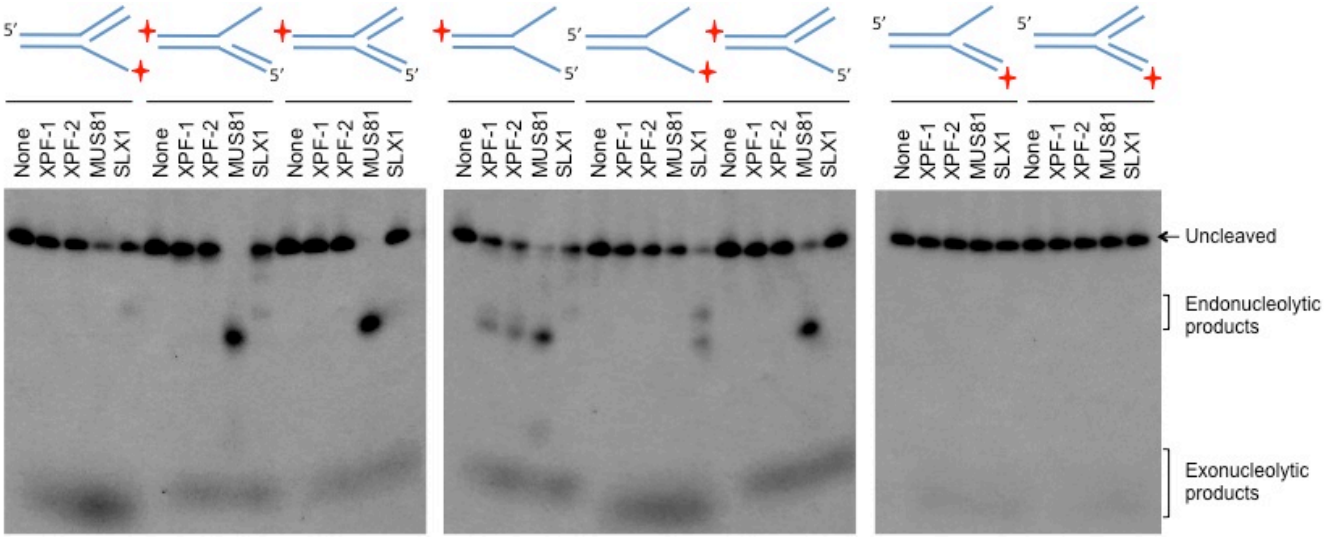

Figure 7. SLX4 associating with different nucleases display different structure-specific incision activity. Recombinant SLX4-nuclease complexes were incubated with different substrates as drawn.

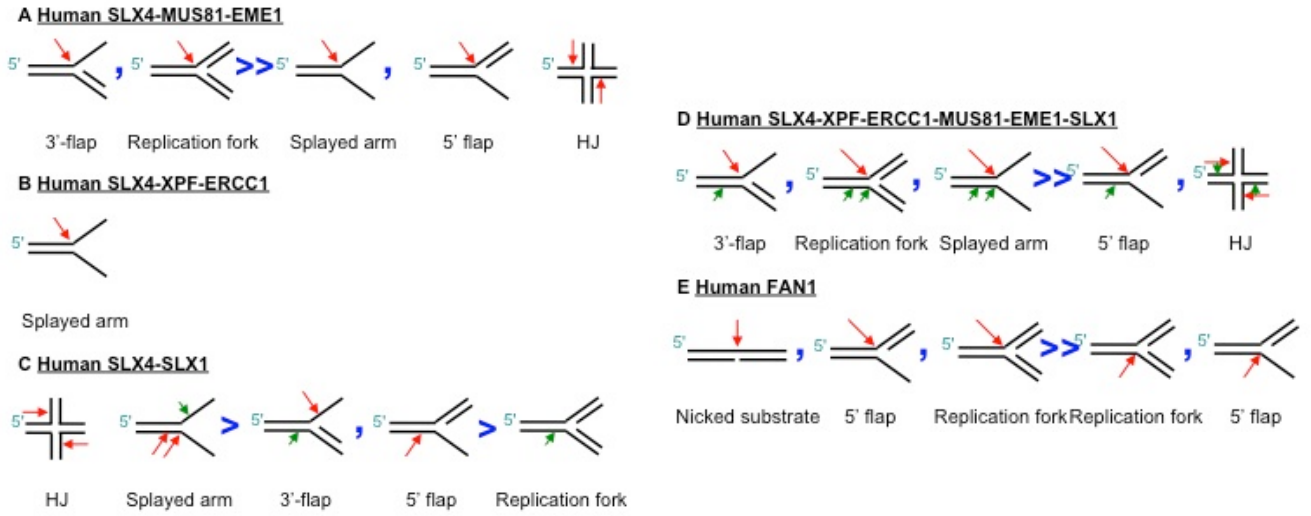

Figure 8. Schematic representations of the substrate specificity of different nucleases. Red arrows represent major activities on indicated substrates. Green arrows indicate minor activities observed.

Task 4: Analysis of incision activity of FLAG-tagged wild type and mutant SLX4 defective in nuclease interaction immunoprecipitated from cells.

I have grown up 293T cells that express tagged wild type or mutant SLX4 that does not interact with either SLX1, MUS81 or XPF. Small-scale anti-FLAG immunoprecipitation was performed on the cell extracts from these cells but the nuclease activity of the immunoprecipitated SLX4-nuclease complexes on DNA substrates appeared to be weak. Work is currently in progress to scale up the immunoprecipitation in order to obtain more active SLX4-nuclease complexes from the cells.

Aim 2: To develop a mammalian cell free system for studying Fanconi anemia-directed ICL repair.

Task 5: Development of soluble cell extract system for studying ICL repair.

HeLa, U2OS, 293T cells and BJ fibroblasts have been acquired from commercial sources. In order to be able to grow up large quantities of BJ fibroblasts, we have transformed the cells with HPV16 E6E7 expression and immortalised them by expression of catalysic subunit of human telomerase. Work is currently in progress to grow up large quantities of each of the cell lines and to make soluble cell extracts.

Task 6: Analysis of ICL repair defect in FA patient cells.

We have transformed and immortalized different FA patient cell lines. Work is currently in progress to grow up large quantities of each of the cell lines.

4. KEY RESEARCH ACCOMPLISHMENTS

- DNA substrates with a site-specific ICL have been successfully generated by click chemistry with high yield. The generation of such substrate by using other systems have been difficult and often with low yield.
- The substrate specificity of different nucleases has been comprehensively documented.

5. CONCLUSION

The majority of the work during the first year has been on generating tools required for addressing the aims of the project. The progress was delayed by unexpected problems of protein insolubility, the presence of contaminating exonucleases in the protein preps and difficulty in generating DNA ICL

substrates at high yield. I have spent much effort and time in scouting for optimal conditions for purifying the contaminating exonuclease away. Additionally, I have spent time on optimising conditions for generating DNA ICL substrates and have now successfully made the substrates. In order to help the progress with protein purification, the laboratory has acquired an FPLC machine. Work is currently in progress using the machine and the optimised conditions determined for purification to produce pure preps of nucleases. Once these nucleases are obtained, *in vitro* incision assays on the DNA substrates will be performed.

With the preliminary analyses of the activities of the different nucleases on the DNA substrates, very different substrate specificities were observed for FAN1 and SLX4-associated nucleases. This suggests that *in vivo*, FAN1 and SLX4-associated nucleases could have redundant roles in dealing with different DNA repair intermediates generated. Our results also showed that SLX4-MUS81-EME1 is extremely active, which is in contrast to the weak activity seen with MUS81-EME1 alone in previous studies (16-18). It indicates that SLX4 can stimulate MUS81-EME1 activity. Most recently, our laboratory and others have reported an essential function of SLX4-MUS81-EME1 and SLX4-SLX1 in Holliday junction resolution important for faithful chromosomal segregation (19-21). The strong activity of SLX4-MUS81-EME1 on replication fork substrates observed in the current *in vitro* work would hint towards additional functions of SLX4-MUS81-EME1 in resolving replication intermediates and hence maintaining cell viability to prevent attrition of hematopoietic stem cells that will ultimately lead to bone marrow failure.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

I will attend the 26th Annual Fanconi Anemia Research Fund Scientific Symposium in Baltimore this year to present work from this fellowship award.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

Nothing to report.

9. OTHER ACHIEVEMENTS

- The laboratory has been awarded an NIH R01 grant, part of which is based on the work from this training award. The funding has allowed the acquisition of an FPLC machine, which will aid the purification of recombinant nucleases.

10. OTHER PROFESSIONAL TRAINING OPPORTUNITIES

- I attended the 25th Annual Fanconi Anemia Research Fund Scientific Symposium where I learnt the latest research progress on Fanconi anemia and presented a poster on my previous work before the current fellowship started.
- I regularly go to seminars at both the Rockefeller University and at the Memorial Sloan Kettering Cancer Center to learn the latest research techniques and progress related to the current work.
- Dr Agata Smogorzewska organises weekly journal clubs and lab meetings that provide important opportunities for keeping up to date with the latest research on Fanconi anemia and other bone marrow failure disorders. I presented every month at these meetings, which are good opportunities for developing my presentation skills.

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12. APPENDICES

The continued approval letter from the IRB is as attached.

SCIENCE FOR THE BENEFIT OF HUMANITY

Institutional Review Board
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March 13, 2014

Agata Smogorzewska, MD, PhD
Laboratory of Genome Maintenance
The Rockefeller University

RE: AAU-0112 Entrance into the International Fanconi Anemia Registry (IFAR) -- REVISED Approval letter (revised approval period)

Dear Dr. Smogorzewska,

Thank you for submitting the modifications to documents for the above protocol (iRIS submission # 323524), as requested in our letter of March 7, 2014. On behalf of the board I have reviewed and approved the modifications.

This study was reviewed and granted stipulated approval at the convened meeting of the Institutional Review Board (IRB) on March 6, 2014. The final approval period is March 12, 2014 through March 11, 2015.

This protocol meets the criteria under 45 CFR 46.404 for research not involving greater than minimal risk for unaffected children involved in this study. This protocol meets the criteria under 45 CFR 46.405 for research involving greater than minimal risk to the individual affected children but presenting the prospect of direct benefit to the individual subjects. Parental permission from one parent is required. Assent must be obtained as outlined in the approved application.

The following documents were reviewed:

Continuing Review Submission Form * This form was part of this submission.	Version 6.1	03/11/2014 03:53 PM EDT
Review Response Submission Form * This form was part of this submission.	Version 17.0	03/11/2014 03:53 PM EDT
Study Application * This application was part of this submission.	Version 1.30	03/11/2014 03:53 PM EDT
Pediatric Assent II - Non Proband	Version 1.5	02/24/2014 07:53 PM EST
Informed Consent	Version 1.7	02/24/2014 07:53 PM EST
Pediatric Assent I- Proband	Version 1.7	02/24/2014 07:53 PM EST
HIPAA	Version 1.0	02/24/2014 07:53 PM EST
2014 Scientific Progress Report	Version 1.0	02/24/2014 07:53 PM EST
Database access agreement form	Version 2.0	02/24/2014

		07:53 PM EST
2014 Study Progress Report * This document was part of this submission.	Version 1.1	03/11/2014 03:53 PM EDT
Cincinnati IRB approval letter	Version 1.1	02/24/2014 07:53 PM EST
Cornell IRB approval letter	Version 1.1	02/24/2014 07:53 PM EST
IFAR Brochure	Version 2.1	02/24/2014 07:53 PM EST
Photo consent form	Version 2.1	02/24/2014 07:53 PM EST
Embedded form	Version 1.1	02/24/2014 07:53 PM EST
Head and neck cancer questionnaire	Version 1.1	02/24/2014 07:53 PM EST
Hematology questionnaire	Version 1.1	02/24/2014 07:53 PM EST
Annual update form	Version 3.1	02/24/2014 07:53 PM EST
IFAR Form	Version 3.2	02/24/2014 07:53 PM EST
Requisition form	Version 2.1	02/24/2014 07:53 PM EST
Enrollment note	Version 2.1	02/24/2014 07:53 PM EST
Release for annual records	Version 2.1	02/24/2014 07:53 PM EST
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Release for research results	Version 2.1	02/24/2014 07:53 PM EST
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Tissue and cell repository agreement	Version 2.1	02/24/2014 07:53 PM EST
Other specimen collection instructions	Version 2.1	02/24/2014 07:53 PM EST
Fibroblast collection instructions	Version 2.1	02/24/2014 07:53 PM EST
Blood collection instructions	Version 2.1	02/24/2014 07:53 PM EST
Research Match E-Mail Language	Version 1.3	02/24/2014 07:53 PM EST
NYS Authorization for Release of Health Info.	Version 1.2	02/24/2014 07:53 PM EST
AAU0112 confidentiality certificate 2013	Version 1.5	02/24/2014 07:53 PM EST
Minn IRB Approval letter	Version 1.5	02/24/2014 07:53 PM EST
CCTS Report	Version 1.5	02/24/2014

Institutional Review Board
Emil C. Gotschlich, MD, Chair
Dale Miller, CIP
IRB Specialist (212) 327- 8411
Lynda Mules, MLA, CIP
IRB Specialist (212) 327-8410
Hospital Bldg., Room 201 Box 331

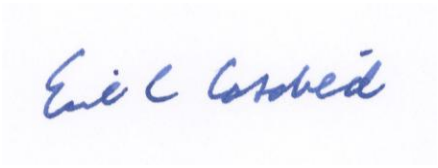
		07:53 PM EST

The number assigned to this protocol **is AAU-0112.**

Please note that submission for Continuing Review of this protocol is due no later than 9:30 am **on February 24, 2015.**

It is the Principal Investigator's responsibility, as required by Federal regulations, to (1) Submit any proposed changes in approved studies to the IRB for review and approval prior to initiation, except where necessary to eliminate apparent immediate hazards to the subjects; (2) To promptly inform the IRB, appropriate institutional officials, the Office for Human Research Protections (OHRP) and the FDA, if applicable of any unanticipated problems involving risks to subjects or others and research related injuries; (3) To obtain the informed consent of the participant in the manner and format approved; and (4) To resubmit to the IRB for continuing review at the interval determined by the IRB to be appropriate to the risk, but not less than once a year.

Sincerely yours,



Signature applied by Emil C Gotschlich on 03/13/2014 12:28:49 PM EDT

Emil C. Gotschlich, MD
Chair, Institutional Review Board
The Rockefeller University
Federal Wide Assurance # 00004658